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A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress

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ABSTRACT

The HAP1 protein (also known as APE/Ref-1) is a bifunctional human nuclear enzyme required for repair of apurinic/apyrimidinic sites in DNA and reactivation of oxidized proto-oncogene products. To gain insight into the biological roles of HAP1, the effect of expressing antisense HAP1 RNA in HeLa cells was determined. The constructs for antisense RNA expression consisted of either a full-length HAP1 cDNA or a genomic DNA fragment cloned downstream of the CMV promoter in pcDNAneo. Stable HeLa cell transfectants expressing HAP1 antisense RNA were found to express greatly reduced levels of the HAP1 protein compared to equivalent sense orientation and vector-only control transfectants. The antisense HAP1 transfectants exhibited a normal growth rate, cell morphology and plating efficiency, but were hypersensitive to killing by a wide range of DNA damaging agents, including methyl methanesulphonate, hydrogen peroxide, menadione, and paraquat. However, survival after UV irradiation was unchanged. The antisense transfectants were strikingly sensitive to changes in oxygen tension, exhibiting increased killing compared to controls following exposure to both hypoxia (1% oxygen) and hyperoxia (100% oxygen). Consistent with a requirement for HAP1 in protection against hypoxic stress, expression of the HAP1 protein was found to be induced in a time-dependent manner in human cells during growth under 1% oxygen. The possible involvement of a depletion of cellular glutathione being linked to the hypoxic stress-sensitive phenotype of the antisense HAP1 transfectants came from the finding that they also exhibited hypersensitivity to buthionine sulphoximine, an inhibitor of glutathione biosynthesis. We conclude that the HAP1 protein is a key factor in cellular protection against a wide variety of cellular stresses, including DNA damage and a change in oxygen tension.

INTRODUCTION

One of the most common lesions generated in DNA is the apurinic/apyrimidinic (AP) site which results from the hydrolysis of the *N*-glycosyl bond linking the base to the deoxyribose moiety. Spontaneous hydrolysis occurs very readily in B-form DNA to the extent that up to 10^4 purines/cell/day may be lost in humans (reviewed in 1,2). This already high spontaneous rate can be increased still further by the action of DNA damaging drugs or radiation. This enhancement in the rate of base loss is mediated either via modification of the DNA base, which increases the lability of the *N*-glycosyl bond, or via enzymatic excision of damaged bases by DNA glycosylase enzymes (reviewed in 3–5). Hence, AP sites are a central intermediate in DNA base excision repair.

Many of the DNA damaging agents which produce AP sites also attack the sugar–phosphate backbone of DNA giving rise to single- or double-stranded breaks. These breaks are not equivalent to those produced by enzymatic cleavage of the DNA backbone, such as that catalyzed by a restriction endonuclease, in that a fragmented deoxyribose group frequently remains linked to the 3′ side of the strand break (3–5). These 3′ deoxyribose lesions block the action of DNA polymerases which require a 3′ OH terminus to prime DNA repair synthesis.

All cells express repair enzymes to remove the AP sites and 3′ blocking sugar lesions generated spontaneously or by cytotoxic drugs and radiation. AP site repair is generally initiated by endonucleolytic cleavage 5′ to the lesion catalyzed by an AP endonuclease, although a second pathway for repair involves cleavage 3′ to the lesion by an AP lyase enzyme (3–5). The 3′ blocking lesions (such as 3′ phosphoglycoaldehyde) induced by DNA damaging agents are removed by a phosphodiesterase enzyme to restore a 3′ OH priming terminus (3–5). In general, these AP endonuclease and 3′ phosphodiesterase activities are contained within a single enzyme. The major AP endonuclease/3′ phosphodiesterase in human cells is termed HAP1 or APE protein (6,7). As well as the aforementioned activities, the versatile HAP1 protein also exhibits RNase H activity (8) and an ability to control the redox state of certain proto-oncogene products such as the

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transcription factor c-Jun (9–11). This latter 'redox' activity is dependent on a cysteine residue in HAP1 which mediates reduction of an oxidized cysteine residue in the DNA binding domain of the target transcription factor (11). The DNA repair and redox activities of HAP1 are distinct both structurally and functionally (11,12).

One physiological stress that many mammalian cells have to tolerate is a change in oxygen tension. High levels of oxygen (hyperoxia) can generate an additional load of highly reactive oxygen derivatives which damage cellular macromolecules, including DNA. A transient decrease in oxygen tension (hypoxia) is a common condition to which many cells of the body are exposed. Indeed, some cell types, such as the endothelial cells lining blood vessels, can be exposed to severe hypoxic stresses as ambient blood oxygen levels drop. It is known that a family of stress proteins is synthesized specifically in response to hypoxia (13–17) which, although of unknown function, are generally assumed to assist in cell survival during times of low oxygen.

We set out to identify whether the HAP1 protein is involved in cellular protection against various stresses, including exposure to DNA damaging drugs and radiation, and changes in oxygen tension. To achieve this, we generated HeLa cell derivatives in which levels of the HAP1 protein were markedly depleted as a consequence of expressing HAP1 antisense RNA. These antisense RNA-expressing transfectants exhibit a significant increase in sensitivity not only to a variety of DNA damaging agents, but also to changes in oxygen tension. Consistent with a protective role for HAP1 in human cells, we also show that the HAP1 protein is induced during hypoxic stress.

MATERIALS AND METHODS

Generation of constructs

The HAP1 cDNA was cloned in both orientations into the mammalian cell expression vector pcDNAneo (Novagen) by excision from pCDM8/HAP1 (6) with *Xho*I and ligation into *Xho*I-digested pcDNAneo. Genomic DNA constructs were generated by subcloning a 7 kb *Hin*dIII fragment of the HAP1 gene (18) in both orientations into the *Hin*dIII site of pcDNAneo.

Cell culture and transfections

HeLa cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 3 mM glutamine and antibiotics. Human umbilical vein endothelial cells (HUVECs) were grown in DMEM containing 10% FBS, heparin (90 U/ml) and basic fibroblast growth factor (1 ng/ml). All cells were grown in tissue culture Petri dishes or flasks (Falcon), at 37°C in a humidified atmosphere containing 5% CO_2.

For determination of growth rates, a defined number of cells was seeded into a multiwell plate and cell numbers counted periodically using a haemocytometer. Transfections were performed by the use of a liposome-mediated DNA transfer technique with DOTAP transfection reagent (Boehringer Mannheim), essentially as described by the manufacturer. Cells were ~60% confluent prior to transfection. Transfectants were selected in medium containing 600 μg/ml G418 (Geneticin; Gibco/BRL).

Isolation of an antiserum specific for the HAP1 protein

A rabbit polyclonal antiserum was isolated following four injections of 200 μg each of purified recombinant HAP1 protein (11). Antisera were screened for specificity by Western blotting against both pure HAP1 protein and HeLa crude cell extracts.

Western blotting

Proteins were separated by SDS–PAGE using the discontinuous system of Laemmli (19), and then electroblotted onto Hybond C membrane (Amersham) at 20 V for 16 h at 4°C, using a Bio-Rad transblot system. After transfer, the membrane was incubated in blocking buffer [Tris-buffered saline/0.05% Tween-20 (TTBS), containing 5% Marvel low-fat milk] for 1 h before exposure to anti-HAP1 antiserum (diluted 1:100 in TTBS) for 1 h. The membrane was then rinsed in TTBS and incubated with ^{125}I-labelled protein A (Amersham) for 1 h before extensive washing in TTBS followed by exposure to X-ray film.

Clonogenic assays

Exponentially growing cells were trypsinized and seeded in 6-well cluster dishes to yield ~200 cells. These were incubated for 5 h to permit attachment to dishes. Cells were then exposed to the appropriate DNA damaging agent or physiological stress, as outlined below. Exposure to hyperoxia was performed in 175 cm^2 flasks.

Cytotoxic drugs

Methyl methanesulphonate (MMS; Sigma) was dissolved in ethanol at 0.1 g/ml. Hydrogen peroxide (Fisons) was diluted in water immediately prior to use. Bleomycin (Lundbeck), mitomycin C and buthionine sulphoximine (BSO; Sigma) were diluted in water and stored at −20°C. Stock solutions of menadione and paraquat (Sigma) were prepared in water immediately prior to use. For MMS, hydrogen peroxide and mitomycin C, exposure of cells to drug was for 1 h before aspiration of the medium, washing in phosphate-buffered saline, and replacement of fresh drug-free medium. A 24 h drug exposure was used for BSO, paraquat, menadione and bleomycin. Cultures were then incubated for ~10 days and surviving colonies counted after fixation and staining with crystal violet. Colonies comprising >50 cells were considered as survivors.

For UV irradiation, the medium was aspirated from cultures and replaced by phosphate-buffered saline (PBS) before irradiation with 254 nm UV light. Following irradiation, the PBS was replaced with fresh growth medium and the cells incubated until visible colonies formed, as described above.

Exposure to hypoxic and hyperoxic growth conditions

Exposure of cells to hypoxia (1% oxygen) was effected by growth in an incubator with automatic oxygen concentration control. At different time points, the cells were removed from the hypoxic incubator and returned to normal growth conditions (normoxia) until visible colonies developed. For hyperoxic treatments, 500 cells were seeded into tissue culture flasks and allowed to adhere for 4 h. The flask was then purged of air by gassing with 100% oxygen, the cap replaced and the cells incubated at 37°C for various times. After this, the 100% oxygen was replaced by gassing the flask with air containing 20% oxygen, and the cells incubated for 10 days for colonies to develop as before.

RESULTS

Isolation of antisense HAP1 RNA-expressing transfectants

Constructs containing the full-length HAP1 cDNA (6) or a fragment of the HAP1 gene (18) cloned in both orientations into

pcDNAneo were generated as described in the Materials and Methods section. These plasmids were designated pHAP1-ac or pHAP1-sc for antisense and sense cDNA constructs, respectively, and pHAP1-ag and pHAP1-sg for the equivalent genomic DNA constructs. A pcDNAneo vector control was also analysed. Following transfection and selection for stable HeLa cell transfectants in medium containing G418, individual colonies were picked and screened by Western blotting for those which had abnormal HAP1 protein levels. To achieve this, a polyclonal antibody specific to HAP1 was raised in rabbits. This antibody specifically recognises a 37 kDa protein on Western blots which co-migrates with purified HAP1 protein (see Figure 1).

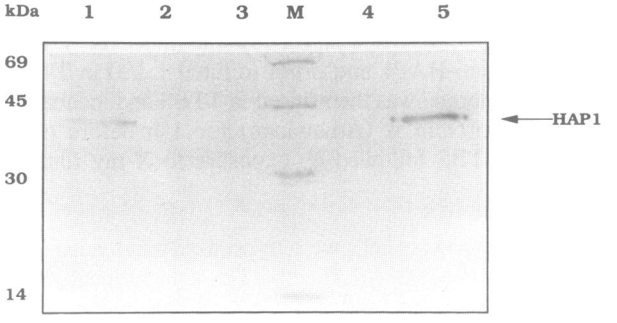

Figure 1. Western blot of whole cell extracts of HeLa cells stably transfected with antisense or sense orientation HAP1 constructs, or the pcDNAneo vector. Lanes: 1, pHAP1-sc; 2, pHAP1-ac (clone 1); 3, pHAP1-ac (clone 2); 4, pHAP1-ag; 5, pHAP1-sg. The sizes of molecular weight standards (lane M) run in parallel are indicated on the left. The position of the 37 kDa HAP1 protein is indicated on the right.

Several examples of both pHAP1-ac and pHAP1-ag transfectants which displayed a stable reduction in HAP1 protein expression were identified. The equivalent pHAP1-sc and pHAP1-sg transfectants, and the vector-only controls, consistently showed equivalent, high levels of HAP1 protein expression. Figure 1 shows Western blotting data for selected HeLa cell clones. An ~10-fold reduction in HAP1 protein levels was seen in the antisense HAP1 RNA-expressing transfectants compared to controls. One clone of each of the antisense, sense and vector-only transfectants was selected for further study. Of the two pHAP1-ac clones shown in Figure 1, clone 1 was selected. All of the selected clones had a similar doubling time (~13.5 h), plating efficiency (~70%) and gross morphology (data not shown).

Depletion of HAP1 confers hypersensitivity to DNA damaging agents in HeLa cells

To test whether depletion of the HAP1 protein in the antisense RNA-expressing transfectants influenced sensitivity to DNA damaging agents, clonogenic survival was measured after

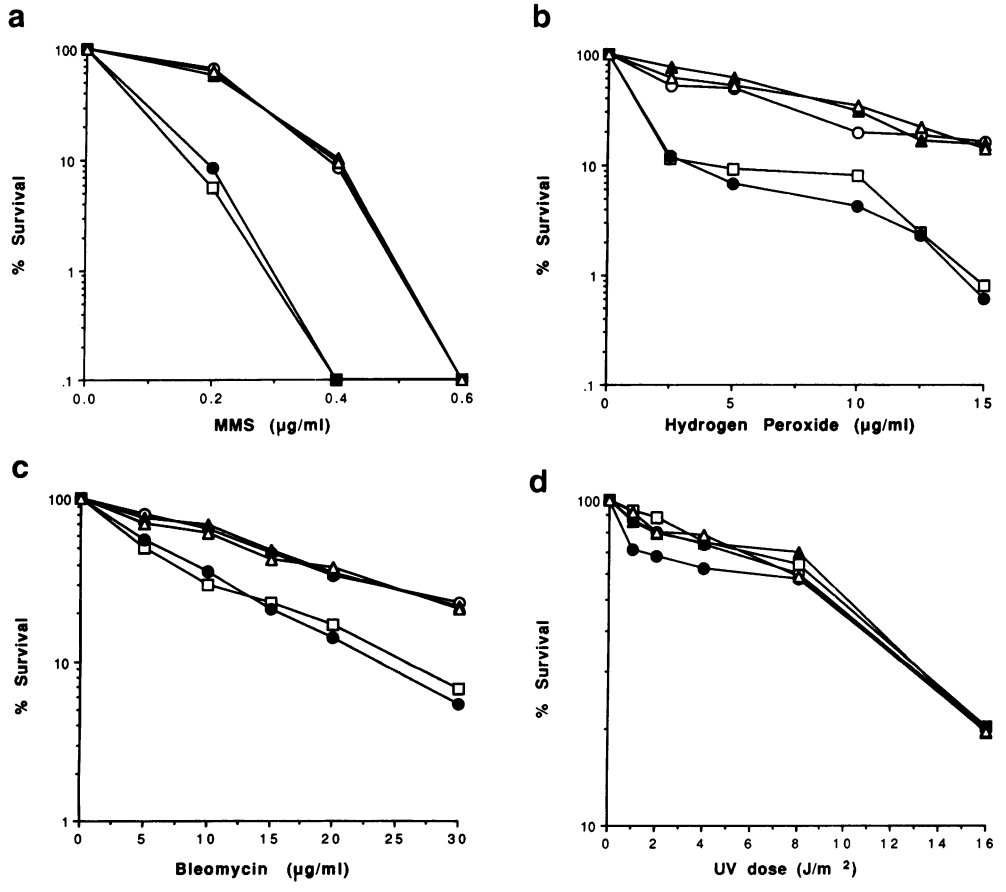

Figure 2. Clonogenic survival curves of HeLa transfectants after exposure to DNA damaging agents. Points represent the mean of two independent determinations. (**a**) MMS; (**b**), hydrogen peroxide; (**c**), bleomycin; (**d**), UV light. Symbols: ○, pHAP1-sc; ●, pHAP1-ac; ▲, pHAP1-sg; □, pHAP1-ag; Δ, pcDNAneo.

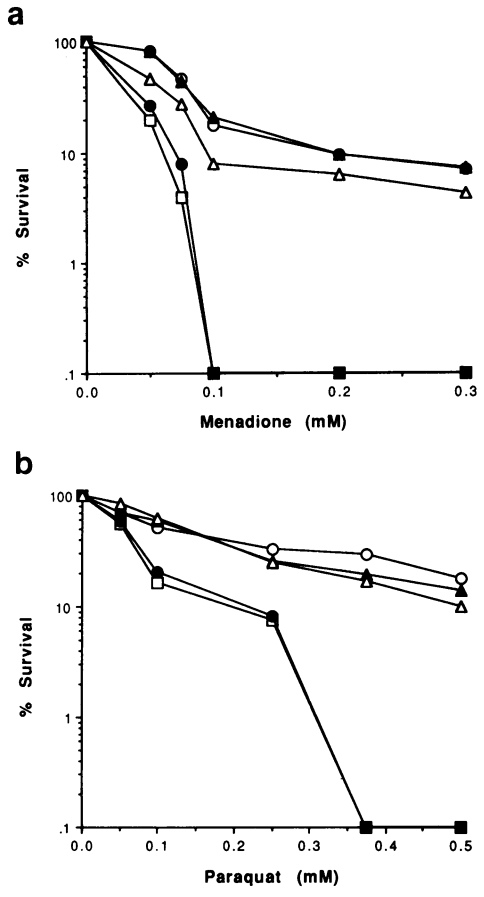

Figure 3. Clonogenic survival curves of HeLa transfectans after exposure to redox cycling drugs. (**a**) Menadione; (**b**) paraquat. Points represent the mean of two independent determinations. Symbols as for Figure 2.

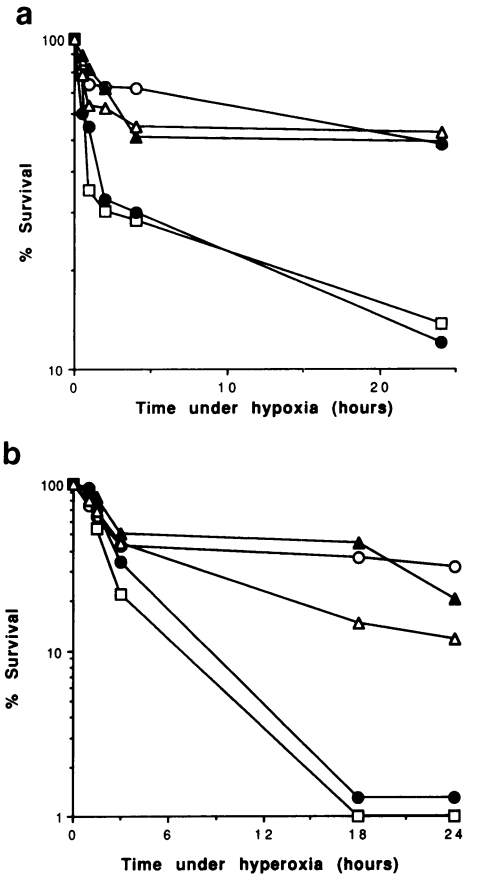

Figure 4. Clonogenic survival curves of HeLa transfectants after growth under hypoxic (**a**) and hyperoxic (**b**) conditions. Symbols as for Figure 2. Points represent the mean of two independent determinations.

exposure of the cells to DNA damaging drugs. In general, agents were selected which generate either AP sites or strand breaks with atypical 3′ termini which might require the action of a dual function AP endonuclease/3′ phosphodiesterase such as HAP1. Figure 2 shows that the antisense HAP1 RNA-expressing transfectants were significantly more sensitive than sense or vector-only controls to MMS, hydrogen peroxide, bleomycin (Figures 2a–c) and mitomycin C (data not shown). However, the antisense cells were not hypersensitive to UV light (Figure 2d). We also tested whether HAP1 was important for cellular protection against compounds which generate reactive oxygen species via redox cycling. Figure 3a and b shows that the antisense transfectants were hypersensitive to killing by two redox cycling drugs, menadione and paraquat.

HAP1 protein is required for cellular protection against changes in oxygen tension

One of the most important stresses to which cells of the human body are exposed is a variation in oxygen tension. While oxygen starvation (hypoxia) may be toxic *per se*, re-oxygenation of hypoxic tissues can also lead to a transient burst of toxic oxygen radical production. We tested, therefore, whether depletion of HAP1 led to an alteration in cellular response to changes in oxygen tension. Figure 4a and b shows that the HAP1 antisense RNA-expressing cells displayed hypersensitivity to the toxic effects of both hypoxia (1% oxygen) and hyperoxia (100% oxygen) in a time-dependent manner.

HAP1 protein levels are elevated during growth under hypoxic conditions

The hypersensitivity of the antisense RNA-expressing cells to hypoxia was particularly striking and occurred within only 2 h of transfer to conditions of low oxygen tension. Such hypersensitivity might have reflected the production of toxic species either while the cells were under hypoxia or while they were being re-oxygenated following transfer to normoxic conditions. We were interested, therefore, to test whether HAP1 protein levels vary in cells during changes in oxygen tension. Figure 5 shows that a time-dependent accumulation of HAP1 protein occurred during exposure of HeLa cells to hypoxic incubation conditions. A similar time-course of HAP1 protein induction by hypoxia was demonstrated in primary human umbilical vein endothelial cells (HUVECs; data not shown).

It has been shown previously that one manifestation of hypoxic stress is a reduction in cellular glutathione content (20). We analysed, therefore, whether exposure to BSO, an agent which inhibits glutathione biosynthesis, would be toxic *per se* to the antisense HAP1 RNA-expressing cells. Figure 6 shows that the antisense RNA transfectants were hypersensitive to killing by BSO in a dose-dependent fashion.

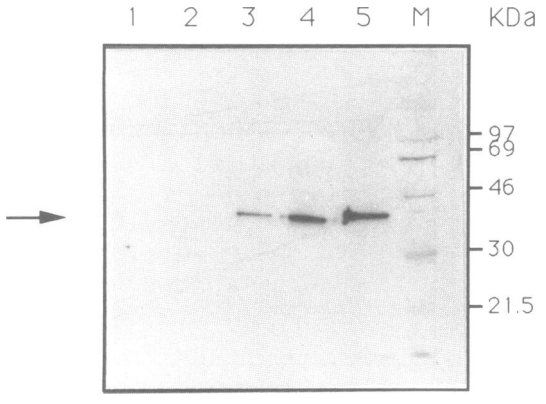

Figure 5. Western blot of HeLa cells after growth under hypoxic conditions for different times. Lanes: 1, time 0; 2, 1 h; 3, 4 h; 4, 8 h; 5, 24 h. The sizes of molecular weight standards (lane M) run in parallel are shown on the right. The arrow on the left indicates the position of the 37 kDa HAP1 protein.

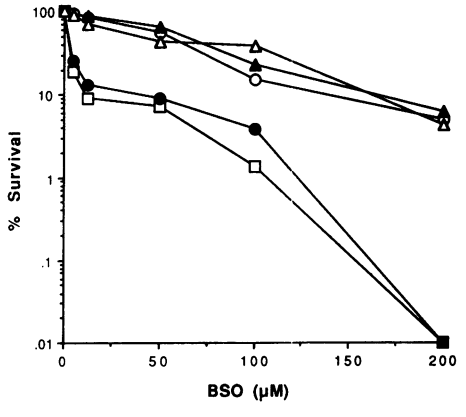

Figure 6. Clonogenic survival curve of HeLa cell transfectants after exposure to BSO. Symbols as for Figure 2. Points represent the mean of two independent determinations.

DISCUSSION

We have shown that antisense RNA-mediated depletion of the DNA repair endonuclease HAP1 from HeLa cells confers hypersensitivity to the effects of both DNA damaging agents and changes in oxygen tension. Consistent with a role in cellular protection against hypoxia, we have also shown that increased levels of the HAP1 protein are found in both transformed (HeLa) and untransformed (HUVEC) cells during exposure to conditions of low oxygen.

The increased sensitivity of the HAP1-depleted cells to DNA damaging agents implies a failure to adequately repair cytotoxic lesions generated by these agents. However, it is formally possible that a failure to adequately maintain the redox status of transcription factors (or indeed other classes of enzymes), which are required to regulate expression of 'survival' genes such as those encoding DNA repair enzymes, could confer hypersensitivity to DNA damaging agents. It will be necessary to distinguish between these possibilities in order to gain insight into the mechanisms by which human cells resist DNA damage stress. The finding that the antisense-expressing cells are hypersensitive to MMS perhaps implicates a deficiency in DNA repair, since this drug directly alkylates DNA and, unlike many of the other agents studied, does not appear to generate reactive oxygen species which might be involved in the inactivation of redox sensitive proteins. Further evidence suggesting that a DNA repair defect underlies the phenotypic alterations observed is that *E.coli* mutants (*xth* and *nfo*) deficient in the major AP endonucleases exonuclease III and endonuclease IV exhibit a similar phenotype to that of the HAP1 antisense-expressing cells, in displaying hypersensitivity to MMS, hydrogen peroxide, bleomycin and paraquat, but wild-type resistance to UV light (21).

We have shown that the HAP1 protein is inducible by hypoxic stress. However, we have failed to demonstrate HAP1 inducibility by DNA damaging agents, including several used in this study (unpublished results). This implies, perhaps, that high level HAP1 protein expression is required to counteract one or more of the cytotoxic species which are generated under hypoxic growth conditions, but not by DNA damaging agents. The nature of this inducing signal, which shows apparent specificity for hypoxic stress, awaits further investigation. One possible candidate which has recently been connected with hypoxic stress is depletion of intracellular reduced glutathione (20), although since many DNA damaging agents also cause depletion of glutathione, it would seem unlikely that the effects of such depletion could be specific for hypoxic stress. It is, however, possible that the extent of glutathione depletion is more severe during hypoxic stress than following exposure to DNA damaging agents. A deficiency in reduced glutathione may have the effect of preventing the detoxification of both endogenous and exogenous cytotoxic species. It will be necessary now to investigate this possibility further, particularly in light of the finding that the HAP1 antisense RNA-expressing cells are hypersensitive to BSO, an inhibitor of glutathione biosynthesis.

It is not possible at this stage to distinguish whether the hypoxia-induced cytotoxicity measured by clonogenic survival assays reflects the production of toxic species generated by hypoxia itself or by the re-oxygenation that accompanies a return to normoxic conditions. Many cells in the body experience transient fluctuations in oxygen tension and it is clear that re-oxygenation of tissues following prolonged hypoxia is highly toxic. What is not in question is that the increased level of HAP1 protein observed during growth under hypoxic conditions requires no contribution from re-oxygenation stress, since the protein samples for analysis of HAP1 inducibility were purified from cells immediately after removal from a 1% oxygen growth environment. Thus, these cells were given no opportunity to substantially re-oxygenate.

A family of proteins induced in mammalian cells by hypoxia have been defined (13–17). Although it is likely that some of these proteins are required for cellular protection against hypoxic stress, their precise role has yet to be defined. At least three of the major hypoxia-inducible proteins are of a molecular weight (35–40 kDa) similar to that of HAP1 (16). As the role of HAP1 in DNA repair and transcription factor function becomes clearer (3,9–12), it may be possible in the future that by an analysis of HAP1 we can understand more fully the underlying reasons for the cytotoxicity of hypoxic stress.

While this paper was in preparation, Ono *et al.* (22) reported the isolation of a rat glioma cell line expressing antisense RNA for APEX nuclease, the rat homologue of HAP1. It was shown

that APEX nuclease depletion from rat cells conferred hypersensitivity to two DNA damaging drugs, MMS and hydrogen peroxide, in agreement with the data presented here for human cells. No other phenotypic changes were reported.

In summary, we have shown that the HAP1 protein makes an important contribution to cellular protection against DNA damaging agents and hypoxic/hyperoxic stresses. The challenge is now to identify which of the activities exhibited by the HAP1 protein is required to mediate a function which is presumably necessary for cell viability in all mammalian species.

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